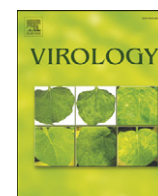


Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Virology

journal homepage: www.elsevier.com/locate/yviro

Overexpression of Toll-like receptor 2/4 on monocytes modulates the activities of CD4⁺CD25⁺ regulatory T cells in chronic hepatitis B virus infection

Ye Zhang^{a,1}, Jian-Qi Lian^{a,1}, Chang-Xing Huang^a, Jiu-Ping Wang^a, Xin Wei^a, Xue-Ping Nan^a, Hai-Tao Yu^a, Li-Li Jiang^a, Xiao-Qin Wang^a, Yan Zhuang^a, Xin-Hong Li^a, Yu Li^a, Ping-Zhong Wang^a, Michael D. Robek^b, Xue-Fan Bai^{a,*}

^a Center of Infectious Diseases, Tangdu Hospital, Fourth Military Medical University, 1 Xin Si Road, Ba Qiao District, Xi'an 710038, Shaanxi Province, China

^b Department of Pathology, Yale University School of Medicine, New Haven, CT 06510, USA

ARTICLE INFO

Article history:

Received 29 July 2009

Returned to author for revision

15 October 2009

Accepted 4 November 2009

Available online 27 November 2009

Keywords:

Hepatitis B virus

Toll-like receptors

Regulatory T cells

innate immune

immunoregulation

ABSTRACT

The significance of TLR expression and Tregs in HBV infection has not been clearly described. In this report, flow cytometry was performed to assess TLR2/4 expression on monocytes and circulating CD4⁺CD25⁺CD127^{low/-} Tregs frequency of 16 acute hepatitis B (AHB), 42 chronic hepatitis B (CHB), 22 asymptomatic HBV carriers (AsC), and 20 normal controls (NC). We found that TLR2 and TLR4 were overexpressed on CD14⁺ monocytes in HBV-infected patients as compared with NCs. Upregulation of TLR2 in NCs and TLR4 in CHBs was observed following HBeAg incubation. However, TLR2 and TLR4 expression decreased after HBeAg stimulation. The difference in the proportion of Tregs between NCs and CHBs was significant. Both Pam3Csk4 (TLR2 agonist)- and lipopolysaccharide (TLR4 agonist)-activated CD4⁺CD25⁺ Tregs showed enhanced suppression function in CHBs. These results suggest that overexpression of TLR2 and TLR4 may modulate the suppressive function of Tregs, which contribute to the immunotolerance of chronic HBV infection.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus that infects ~350 million people worldwide, with a particularly high prevalence in Asia and Africa (Lok and McMahon, 2001). HBV infection in adults frequently results in a self-limiting, acute hepatitis that confers protective immunity and causes no further disease. In contrast, most children fail to clear the virus, resulting in chronic infection. Most chronically infected patients remain largely asymptomatic without life-threatening liver disease, but 1–2 million people die annually due to the consequences of chronic HBV infection, including liver cirrhosis and hepatocellular carcinoma (Hoofnagle et al., 2007; Lok and McMahon, 2001). Patients often exhibited impairment of HBV-specific T cell activity and failed to generate sufficient cellular immunity against the virus during chronic HBV infection (Chisari and Ferrari, 1995; Webster et al., 2004). However, the precise mechanism associated with T cell tolerance is still not completely understood due to the host range limitations of HBV and the lack of small animal models.

With regard to the role of CD4⁺CD25⁺ regulatory T cells (Tregs) in viral persistence, an abundance of experimental data has suggested that CD4⁺CD25⁺ Tregs activities might represent the escape mechanisms responsible for virus-induced immune modulation. During some virus

infections, CD4⁺CD25⁺ Tregs responses appear to cause viral persistence and chronic infection, particularly in human immunodeficiency virus (HIV) (Kinter et al., 2004) and hepatitis C virus (HCV) infection (Boettler et al., 2005). According to more recently published data, CD4⁺CD25⁺ Tregs could contribute to a sustained state of virus-specific T cell collapse, which is characteristic of chronic HBV infection (Barboza et al., 2007; Franzese et al., 2005; Kondo et al., 2006; Peng et al., 2008; Stoop et al., 2005; Xu et al., 2006; Yang et al., 2007). However, the mechanism for modulation of CD4⁺CD25⁺ Tregs functions during chronic HBV infection remains obscure. Because Tregs are naturally occurring, it is anticipated that the cells would be strongly influenced by innate immunity, including the activation of Toll-like receptors (TLRs) (Liu et al., 2006a; Medzhitov, Preston-Hurlburt, and Janeway, 1997).

TLRs, which are commonly expressed on monocytes, play a crucial role in early host defense. Activation of TLRs induces the expression of some kinds of cytokines and chemokines, which control activation of adaptive immune responses (Akira and Takeda, 2004). An abundance of experimental data also suggested that alterations in TLRs by virus might be responsible for virus-induced immune modulation. Wu et al. found that murine nonparenchymal liver cells can be activated by TLR3 and TLR4 to produce interferon-β (IFN-β) and other mediators that can potentially suppress HBV replication (Wu et al., 2007).

Recent studies have demonstrated that the immunosuppressive function of CD4⁺CD25⁺ Tregs can be modulated via TLR signaling. Various pathways, including the direct effects of TLRs on CD4⁺CD25⁺ Tregs as well as the indirect route through antigen presenting cells and

* Corresponding author. Fax: +86 29 83537377.

E-mail address: xfbai@fmmu.edu.cn (X.-F. Bai).

¹ These authors contributed equally to this work.

related cytokine productions, may collectively contribute to the generation, expansion and function of CD4⁺CD25⁺ Tregs (Liu and Zhao, 2007). Nevertheless, the effect of TLRs to CD4⁺CD25⁺ Tregs is still somewhat controversial. TLR2, TLR4, and TLR8 is generally thought to downregulate the function of Treg (Pasare and Medzhitov, 2003; Peng et al., 2005; Suttmuller et al., 2006). But new evidence also suggested that LPS treatment increases the suppressive ability of CD4⁺CD25⁺ Tregs, and LPS-activated CD4⁺CD25⁺ Tregs can efficiently control the occurrence of naïve CD4⁺ effector T cell-mediated diseases (Caramalho et al., 2003). Because the findings of different studies have been contradictory, controversy remains as to whether changes in the frequency and function of circulating TLRs, CD4⁺CD25⁺ Tregs, and their interactions are correlated with HBV clearance and persistence.

Data reported here suggest the following scenario. To establish a chronic infection, HBV may make use of its antigens to change the expression profiles of TLR2 and TLR4 on monocytes. HBV antigens also influence the frequency and function of CD4⁺CD25⁺ Tregs, whose suppressive activity enhanced when exposure to TLR2 and TLR4 agonists, to contribute to immune dysfunction and account for viral persistence. Our results therefore suggest a potential mechanism that TLR2/4 signaling in regulating the adaptive immune response may contribute to chronic HBV infection.

Results

Overexpression of TLR2 and TLR4 on monocytes from HBV-infected patients

We examined peripheral blood monocytes from 42 patients with HBeAg-positive chronic hepatitis B (CHB) 16 patients with acute hepatitis B (AHB), 22 HBeAg-positive asymptomatic HBV carriers (AsC), and 20 normal controls (NC). The clinical data obtained for the enrolled subjects are listed in Table 1. Separate CD14-positive populations of cells were gated by flow cytometry (Fig. 1A). The level of TLR2 and TLR4 expression was measured in these cell populations, and a typical flow histogram of CD14-positive TLR expression is shown in Fig. 1B. Summary data from the enrolled volunteers are shown in Fig. 1C.

Mean fluorescence intensity (MFI) values corresponding to TLR2 were upregulated in AHB, CHB, and AsC groups compared with NC. The MFI \pm standard deviation (SD) values were NC = 96.79 \pm 16.84, AHB = 130.09 \pm 35.37, CHB = 212.92 \pm 81.72 and AsC = 161.13 \pm 43.31. The MFI value evaluated for CHB patients was significantly increased compared to those of AHB patients ($P < 0.001$) and AsC patients ($P = 0.011$). A significant difference was also observed between AHB and AsC patients ($P = 0.017$).

The MFI values corresponding to TLR4 were also increased in AHB, CHB and AsC groups compared with NC. The MFI \pm SD values were

NC = 19.73 \pm 5.44, AHB = 28.07 \pm 5.39, CHB = 24.74 \pm 8.17 and AsC = 28.96 \pm 5.29. The results revealed no significant differences between AHB and CHB and between AHB and AsC ($P > 0.05$).

Influence of HBeAg and HBcAg on TLR2 and TLR4 expression

To explore whether TLR2 and TLR4 could be specifically affected by HBV antigen *in vitro*, PBMCs from 9 of NCs and 14 of CHBs were cultured for 20 h in RPMI 1640 supplemented 10% FBS, with or without (untreated control) 2 μ g/mL of purified HBeAg or 1 μ g/mL of purified HBcAg, and analyzed for co-expression of CD14 and TLR2/TLR4 by flow cytometry. Doses of 1 μ g/mL, 2 μ g/mL, 5 μ g/mL, and 10 μ g/mL were tested for each antigen and optimal response was observed with 2 μ g/mL of purified HBeAg or 1 μ g/mL of purified HBcAg. NC individuals did not demonstrate significant changes in either TLR2 (94.78 \pm 14.76 vs. 90.38 \pm 18.10, $P = 0.164$) or TLR4 (17.05 \pm 6.70 vs. 16.05 \pm 7.23, $P = 0.129$) expression in response to HBcAg stimulation (Figs. 2A and B). In contrast, Figs. 2C and D show that decreased expressions of TLR2 (155.79 \pm 37.63 vs. 128.06 \pm 23.81, $P = 0.0067$) and TLR4 (23.11 \pm 3.93 vs. 19.51 \pm 3.52, $P = 0.0002$) were observed in CHBs following HBcAg incubation. Simultaneously, upregulation of TLR2 expression in NCs (94.78 \pm 14.76 vs. 120.42 \pm 17.11, $P = 0.0039$) and TLR4 expression in CHBs (23.11 \pm 3.93 vs. 32.07 \pm 8.35, $P = 0.0001$) were detected following HBeAg stimulation. In contrast, No significant differences in TLR4 expression were noticed for NC individuals (17.05 \pm 6.70 vs. 23.40 \pm 12.37, $P = 0.074$) or in TLR2 for CHBs (155.79 \pm 37.63 vs. 171.81 \pm 41.27, $P = 0.153$).

We subsequently studied the variation in CD14⁺, CD14⁺TLR2⁺, and CD14⁺TLR2⁺ cells in the PBMC population. A representative PBMC sample from CHBs analyzed by flow cytometry is shown in Fig. 2E. Interestingly, after incubations for 20 h with HBeAg or HBcAg, the proportions of CD14⁺ and CD14⁺TLR2⁺ cells in the PBMCs population were significantly decreased for both NCs and CHBs (Figs. 2F and G). A significant increase in CD14⁺TLR2⁺ cells was observed following HBeAg stimulation, but neither NCs nor CHBs demonstrated significant differences in response to HBcAg (Fig. 2H).

Increased frequency of circulating CD4⁺CD25⁺CD127^{low/-} Tregs in CHB patients

We also analyzed peripheral blood from the volunteers described above to determine the percentage of CD25⁺ T cells in the total CD4⁺ T cell population. In CHB patients, the CD4⁺CD25⁺CD127^{low/-} (fluorescence intensity of CD127 $< 10^2$) population represented 3.93 to 8.76% of CD4⁺ T cells; however, the total CD4⁺ CD25⁺ T cell population comprised 6.60 to 28.87% of CD4⁺ T cells (Figs. 3A and B). As a result, the frequency of CD4⁺CD25⁺CD127^{low/-} Tregs was significantly higher in CHB patients (mean \pm SD: 6.00 \pm 1.24%) compared to NC (mean \pm SD: 4.70 \pm 1.30%) ($P = 0.0048$), but not compared to AHB

Table 1
Clinical characteristic of studied subjects.

Group	AHB	CHB	AsC	NC
Case	16	42	22	20
Sex (males/female)	10:6	28:14	15:7	13:7
Age (years)	29.3 \pm 7.5	27.5 \pm 7.8	27.9 \pm 9.1	29.3 \pm 4.3
ALT (U/L)	>400 (1222 \pm 679)	>80 (149 \pm 28)	<40 (25.5 \pm 8.1)	N.A.
HBeAg positive	16	42	22	0
HBeAg positive	9 (56.25%)	42	22	0
HBeAb positive	7 (43.75%)	0	0	0
HBcAb positive	16	42	22	0
HBcAb IgM positive	16	0	0	0
HBV DNA positive	16	42	22	0
genotype B	5	13	6	0
genotype C	0	2	0	0
genotype B+C	11	27	16	0

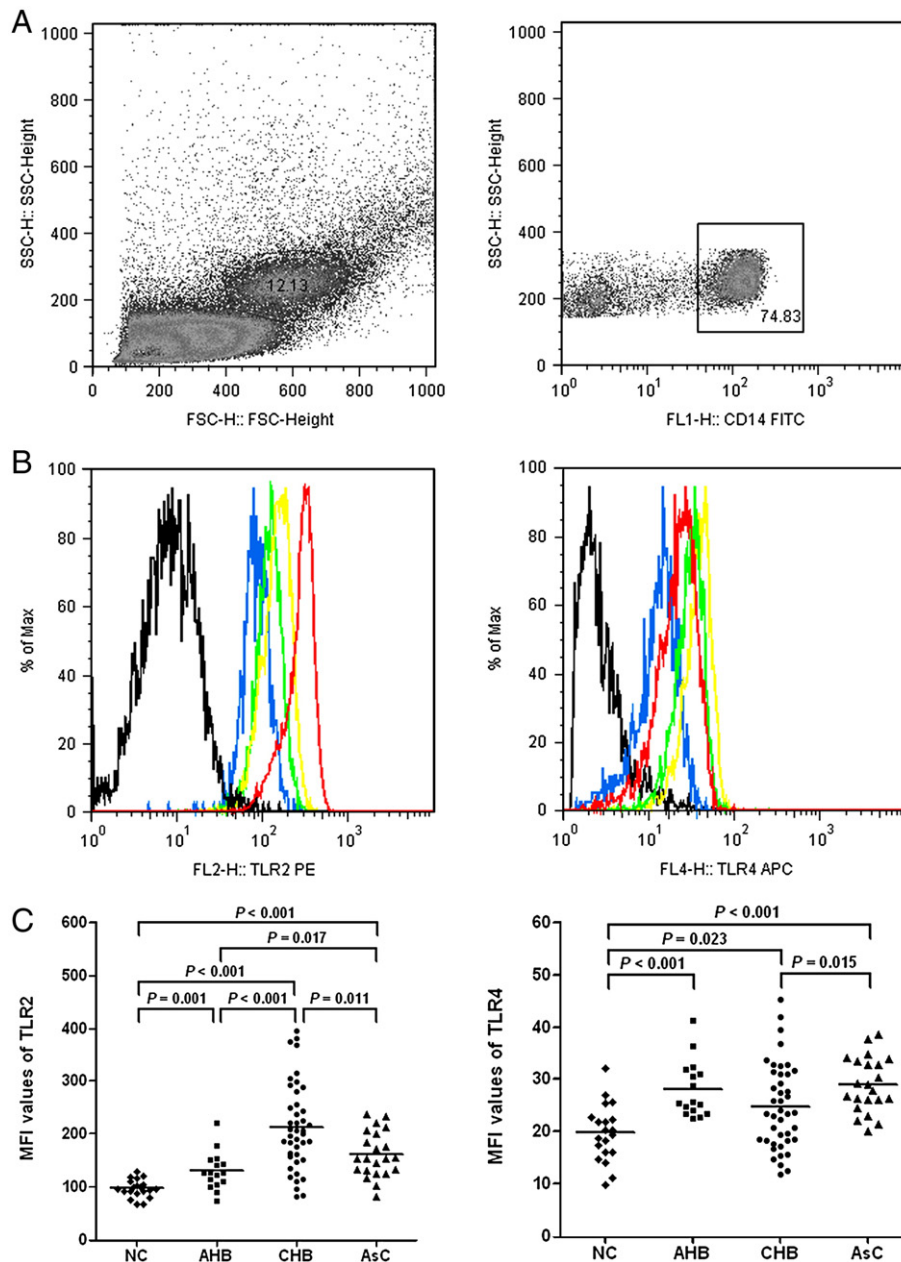


Fig. 1. Typical TLR profiles and expression on CD14⁺ peripheral blood monocytes obtained from control subjects and patients with HBV infection. (A) PBMCs from a representative patient with CHB were separated using the gates shown by flow cytometry. The larger gated population is made up of monocytes, while the smaller cells were lymphocytes. Peripheral blood monocytes were stained with directly conjugated antibodies specific for CD14 and either TLR2 or TLR4. (B) Diagrams represent 10,000 CD14⁺ gated cells. The black line represents expression of the isotype control, the blue line the control subject, the green line the AHB patient, the yellow line the AsC patient, and the red line the CHB patient. (C) TLR2 and TLR4 expression on CD14⁺ monocytes in various subjects. The horizontal bars indicate the median level of TLR2 or TLR4. The individual expression level for each subject included in the analysis is shown. Significances of differences were calculated using the Dunn's multiple comparison test.

patients (mean \pm SD: $5.32 \pm 1.84\%$) ($P=0.55$) or AsC patients (mean \pm SD: $5.42 \pm 1.35\%$) ($P=0.45$). Comparison of circulating CD4⁺CD25⁺CD127^{low/-} Treg frequencies among other groups revealed no significant differences (Fig. 3C). However, there was no significant difference in the frequency of the total CD4⁺CD25⁺ T cell population between groups (Fig. 3D; $P=0.073$; Kruskal–Wallis H -test).

Factors related to TLR expression and Treg among CHB patients

To investigate whether the circulating TLR2/4 and CD4⁺CD25⁺CD127^{low/-} Tregs were correlated with the HBV replication level, we measured the viral titers of serum from all tested HBV-infected individuals. In CHB patients, Spearman analysis showed that TLR4 expression negatively correlated with the frequency of CD4⁺CD25⁺

CD127^{low/-} Tregs ($r = -0.501$, $P = 0.0007$, Fig. 4A), but there was no correlation between Tregs and serum copies of HBV DNA ($r = 0.243$, $P = 0.121$, Fig. 4B).

HBV genotypes were determined by nested-PCR using type-specific primers, as described previously (Naito, Hayashi, and Abe, 2001) for all enrolled HBV infected patients. Among the 80 patients, 24 (30%) were genotype B, 2 (2.5%) were genotype C and 54 (67.5%) were mixed genotype B and C. No A, D, E, or F genotypes were found. This is in agreement with the characteristic geographic distribution of HBV genotypes in China. There were no significant difference in ALT values, positive rate of HBeAg, and HBV DNA value among the different genotypes (data not shown). Among 42 of CHB patients, 13 (30.95%) were genotype B, 2 (4.76%) were genotype C, and 27 (64.29%) were mixed genotype B and C. For the different genotypes

among CHB patients, neither TLR2/4 expression nor Tregs frequencies showed any significant differences (Fig. 4C).

TLR2 and TLR4 enhance the suppression function of CD4⁺CD25⁺ Tregs

To investigate the effect of CD4⁺CD25⁺ Tregs on responder cells and to determine the direct and indirect modulation of TLR2/4 on CD4⁺CD25⁺ Tregs, PBMCs-Tregs (depletion of Tregs) and PBMCs-Tregs + Tregs (depletion of Tregs and reconstituted Tregs at a ratio of 1:1) were used as effector cells under stimulation with anti-CD3 or HBsAg, respectively. Both PBMCs-Tregs and PBMCs-Tregs + Tregs proliferated strongly in response to anti-CD3 but a little weakly in response to HBsAg stimulation. Adding CD4⁺CD25⁺ Tregs partially suppressed (more than 20%) PBMCs proliferation stimulated by both anti-CD3 and HBsAg (Fig. 5, lanes 1 and 2). When PBMCs-Tregs were cocultured with Tregs which had been pretreated with Pam3Csk4 (TLR2 agonist) and LPS (TLR4 agonist), cellular proliferation was significantly decreased under both anti-CD3 and HBsAg

stimulation (Fig. 5, lanes 3 and 4). Furthermore, when PBMCs-Tregs were pretreated with Pam3Csk4 and then cocultured with autologous Tregs, neither anti-CD3 nor HBsAg stimulation showed significant effects on cellular proliferation (Fig. 5, lane 6). Interestingly, LPS-activated PBMCs-Tregs significantly decreased cellular proliferation when cocultured with Tregs stimulated by anti-CD3 but not by HBsAg (Fig. 5, lane 5).

Discussion

In the current study, we characterized the expression levels of TLR2 and TLR4 proteins on CD14⁺ monocytes during acute and chronic HBV infection and observed that monocytes from patients with HBV infection expressed significantly higher levels of TLR2 and TLR4 proteins compared with those from normal controls, which consistent with earlier data obtained for TLR2 and TLR4 expression on hepatocytes from CHB patients (Guo, Wei, and Yang, 2007; Wei et al., 2008). Because TLR2 and TLR4 were detected as both the important

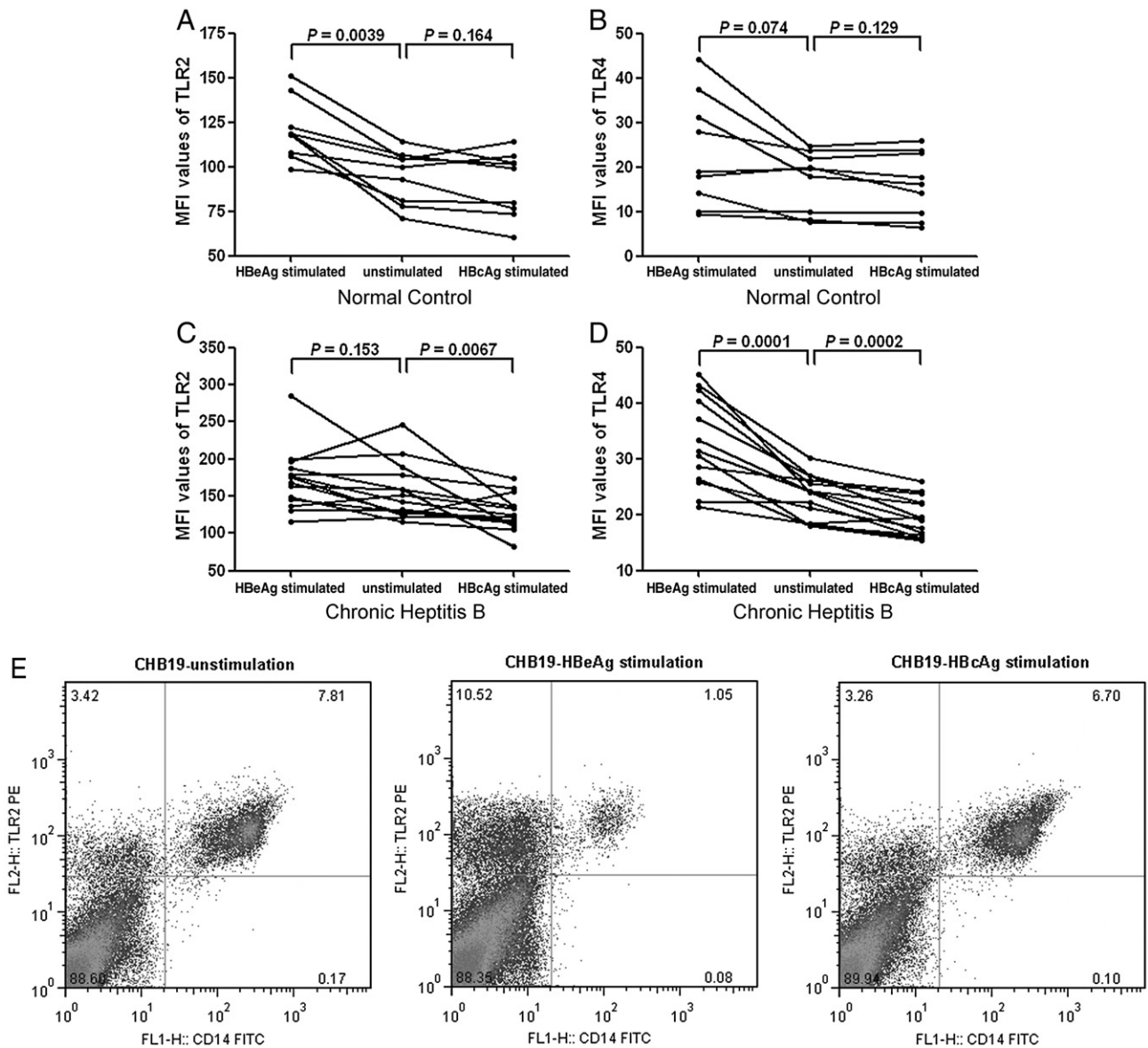


Fig. 2. Variations in TLR2 and TLR4 expression and proportions of CD14⁺, CD14⁺TLR2⁺, and CD14⁺TLR2⁺ cells in response to HBeAg and HBcAg stimulation in NCs and CHBs. Wilcoxon matched pairs tests were used to assess TLR2 and TLR4 expression on CD14⁺ monocytes from NCs and CHBs following incubation with HBeAg or HBcAg for 20 h (A–D). A typical PBMC sample from CHBs analyzed by flow cytometry is shown in E. The proportion of CD14⁺, CD14⁺TLR2⁺ and CD14⁺TLR2⁺ cells present in PBMCs (F–H) after HBV antigen stimulation using Wilcoxon matched pairs tests.

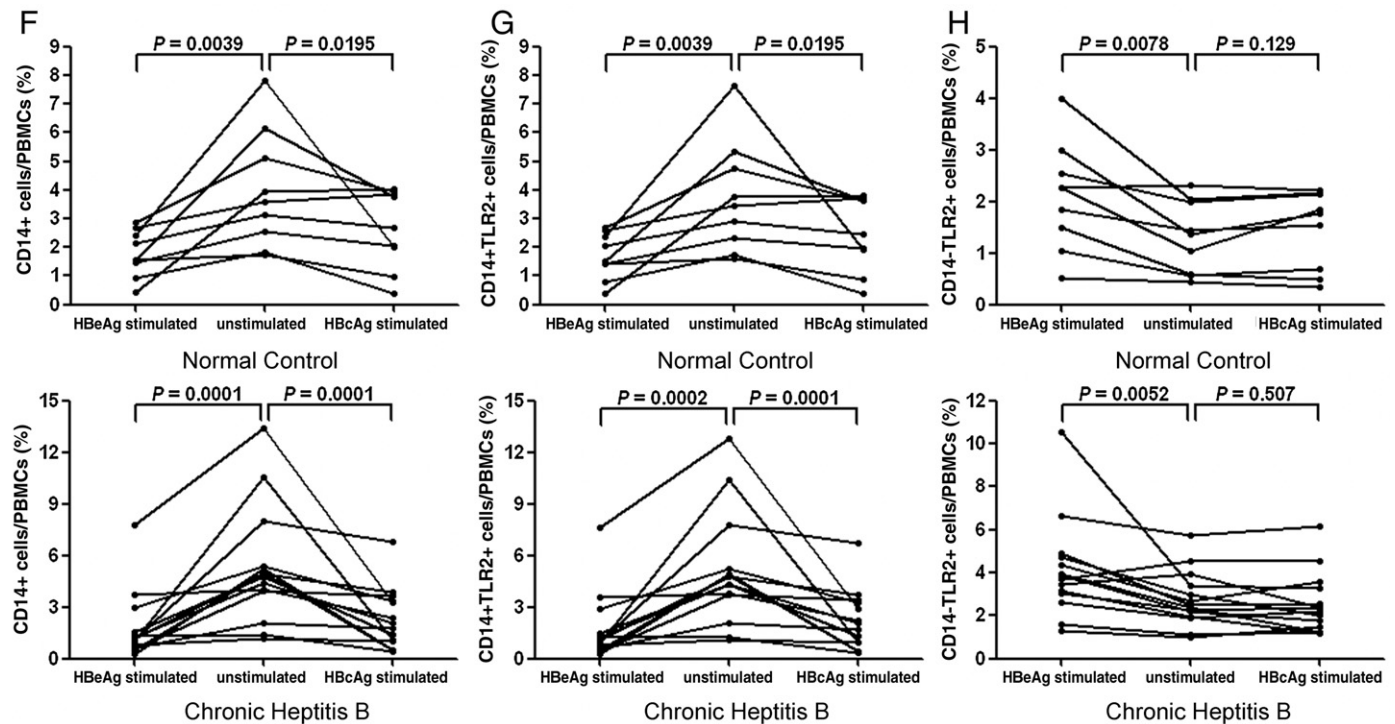


Fig. 2 (continued).

components of the innate immunity in humans and microbial recognition receptors, our works and others suggested that HBV infection could modify the innate immune by upregulating the expression of TLR2 and TLR4, which may also play an important role in recognition and initiation of HBV infection.

In contrast to our findings, others (Chen et al., 2008; Riordan et al., 2006; Visvanathan et al., 2007) reported that HBV downregulates the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes, whereas the level of TLR4 expression did not significantly differ in response to HBV infection. To validate our results, we investigated the postulate that the immunodysregulation of TLR2 and TLR4 associated with HBV infection is partially mediated by stimulation of HBV-encoded antigens. Purified HBcAg and HBeAg were used to stimulate isolated PBMCs from NCs and CHBs, and their effect on TLR2 and TLR4 expression was determined. We found that TLR2 expression was increased by HBeAg induction in normal individuals but no significant changes were detected in CHBs. It is known that pre-C/C mRNA is one of the first transcripts to be transcribed and translated following establishment of infection, thereby making HBeAg as an early-phase viral protein product of HBV infection in the serum (Yuh, Chang, and Ting, 1992). Therefore, during the early phase of infection, HBeAg may function as a major target for the inflammatory immune response and induce increased expression of TLR2. Once chronic infection has been established, the continual secretion of HBeAg may serve as the tolerogen, which is necessary to maintain the tolerant state. Therefore, no TLR2 response following HBeAg stimulation was detected in CHBs. Our results indicate that HBeAg may function as both an immunogen (early stage of infection) and a tolerogen (persistent infection) depending on the context of HBV infection. Interestingly, we observed that following either HBeAg or HBcAg incubation, the proportion percentages of CD14⁺ and CD14⁺TLR2⁺ cells in PBMCs were decreased in both NCs and CHBs. This may be due, in partly, to the toxicity of the purified antigens. In our preliminary experiments, difference doses of antigens were tested, and an optimal response was observed with 2 µg/mL of HBeAg and 1 µg/mL of HBcAg, as a high dose of stimulus (10 µg/mL) resulted in great cell death. Our results also reveal that TLR4 showed

contradictory expression profiles in response to HBcAg and HBeAg during chronic HBV infection. This result has the remarkable implication that an immunogenic difference between HBcAg and HBeAg exists. In addition, we also determined the HBV genotypes of the enrolled patients and found that the majority of them were genotype B and C, differing from Oceania, which exhibits predominantly genotype A. We presumed that the differences between our findings and those of others may be due to variations in geographic location, viral subtypes and host genetic background.

Our study did not reveal a significant difference in total CD4⁺CD25⁺ T cells in the peripheral blood of HBV-infected patients. Recently, it has been suggested that there is an inverse relationship between the expression of Foxp3 and CD127, and in combination with surface expression of CD25 it can distinguish between human regulatory and conventional CD4⁺ T cells in human blood (Hartigan-O'Connor et al., 2007; Liu et al., 2006b). Latest study also showed that 88.1–96.1% of CD25⁺CD127^{low/-} T cells expressed Foxp3 in the peripheral blood of gastric cancer patients (Shen et al., 2009). We have used this information to analyze the Treg population within total CD4⁺CD25⁺ T cells and show that a smaller population of CD4⁺CD25⁺CD127^{low/-} Tregs was significantly increased in CHB patients compared with healthy controls. Our data together with those of others (Barboza et al., 2007; Franzese et al., 2005; Kondo et al., 2006; Peng et al., 2008; Stoop et al., 2005; Xu et al., 2006; Yang et al., 2007) imply that Tregs may be involved in the mechanism responsible for viral persistence. Further analysis did not reveal a positive correlation between the frequency of circulating CD4⁺CD25⁺CD127^{low/-} Tregs and the serum HBV DNA load. Those results are identical to that reported by Franzese and Stoop (Franzese et al., 2005; Stoop et al., 2005). It may indicate that the early stage infection (first few days/weeks after infection) really determining the total number of Tregs and possible disease outcome. It is also possible that Tregs accumulate and expand locally at the site of infection, where they exert their suppressive activity (Cao et al., 2003; Yamazaki et al., 2003). Another possibility is that a population of HBV-specific regulatory cells that differ from CD4⁺CD25⁺CD127^{low/-} Tregs might be induced during chronic HBV infection, as in Barboza's

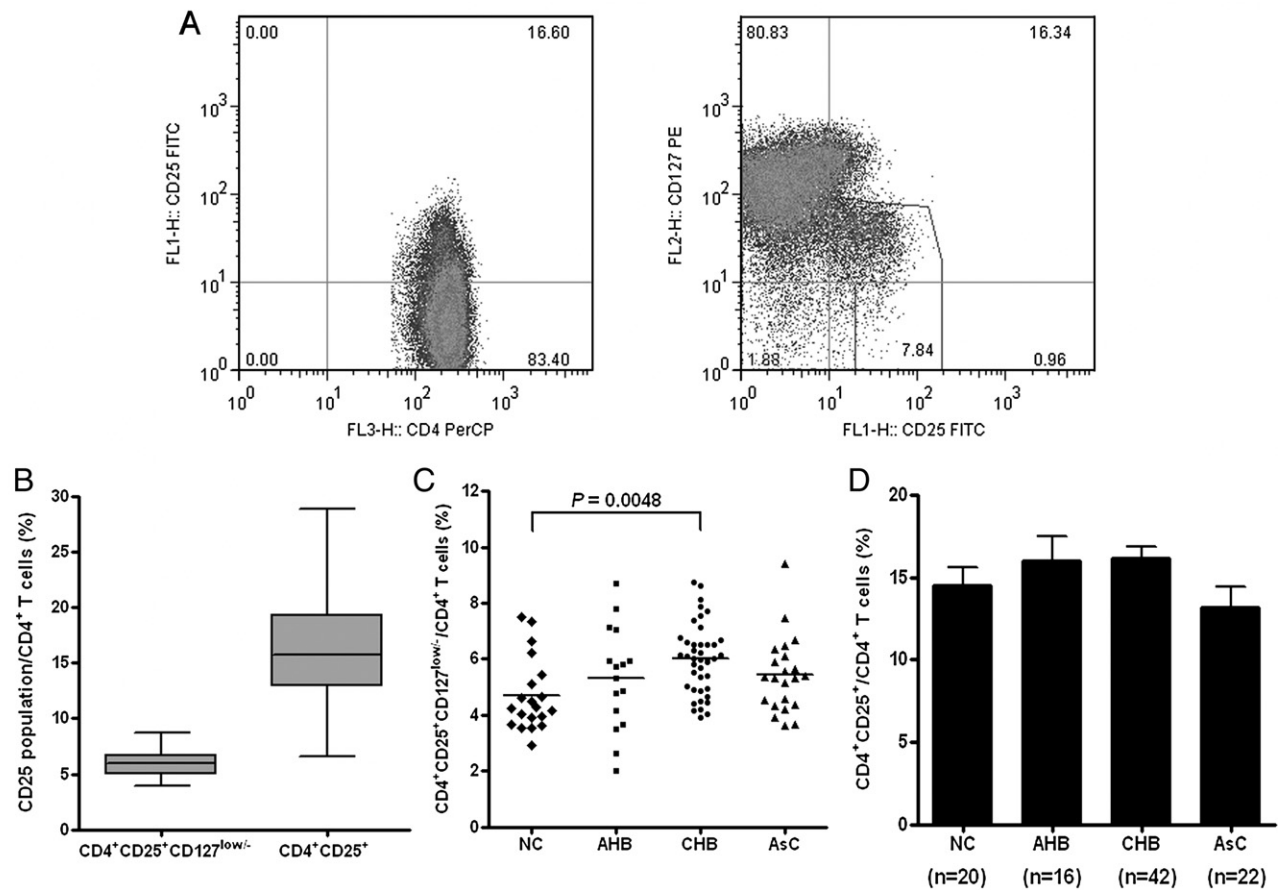


Fig. 3. Frequency of circulating CD4⁺CD25⁺CD127^{low/-} regulatory T cells (Tregs) in various subjects. (A) CD4⁺ T cells were separated into CD25⁺ and CD25⁻ T cell subsets, and CD4⁺CD25⁺CD127^{low/-} Tregs were defined by the fluorescence intensity of CD25 and CD127 obtained using isotype control antibody. (B) Frequency of CD4⁺CD25⁺CD127^{low/-} and total CD4⁺CD25⁺ T cells from chronic hepatitis B (CHB) patients. Data are expressed as box plots in which the horizontal lines represent the 25th, 50th, and 75th percentiles of the measured frequencies of CD4⁺CD25⁺ T cells and Tregs. (C) Percentages of CD4⁺CD25⁺CD127^{low/-} Tregs in various subjects. The horizontal bars indicate the median percentage of Tregs. The individual frequency for each subject included in the analysis is shown. Significance of differences was calculated using the Dunn's Multiple Comparison test. (D) The percentage of total CD4⁺CD25⁺ T cells for the different groups.

study, a CD4⁺FoxP3⁺ IL-10-producing cell population in response to HBcAg contribute to maintain active viral replication and subvert host immune response (Barboza et al., 2007). In addition, virus may potentially effects expression of these genes, since virus-specific Tregs displayed a distinct phenotype and CD127 and CD25 expression defines CD4⁺ T cell subsets are differentially in HCV and HIV infection (Dunham et al., 2008; Heeg et al., 2009). However, measles virus infection in adults may not show this phenomenon due to Yu's study. Moreover, measles virus infection not only up-regulates CD4⁺CD25⁺CD127^{low/-} Tregs, but also induces IL-10-producing CD14⁺ and CD4⁺CD25⁺ cells in PBMCs (Yu et al., 2008). Further studies will determine whether monitoring the three subsets of CD4⁺ T cells defined based on the expression of CD25 and CD127 should be used in the clinical management of HBV-infected individuals and the levels of IL-10 expression should be determined in these cells.

Recent study in autoimmune liver diseases (AILD) has focused on the role of monocytes and their relationship with Tregs. Longhi et al. (2009) found that monocytes were higher in number and expressed higher levels of TLR4. Addition of Tregs in AILD enhanced monocyte migration, magnified TNF- α over IL-10 production and markedly increased TLR4 expression levels. Monocyte overactivation and inability of Tregs to restrain it may contribute to the loss of immune tolerance and perpetuation of the autoimmune attack in AILD. We observed that exposure of CD4⁺CD25⁺ Tregs to both TLR2 ligand Pam3Csk4 and TLR4 ligand LPS could enhance the immunosuppressive function and decrease cellular proliferation in patients with

CHB. These findings demonstrated that CD4⁺CD25⁺ Tregs may respond directly to TLR2/4 signaling, a mechanism that is likely to contribute to viral persistence and chronic HBV infection. In contrast, increased inhibitory capability of CD4⁺CD25⁺ Tregs in response to LPS-activated PBMCs-Tregs was found under non-specific anti-CD3 stimulation but not HBsAg-specific challenge. Preincubation of PBMCs-Tregs with Pam3Csk4 did not affect the function of CD4⁺CD25⁺ Tregs. These data suggested that indirect regulatory effects of TLR2/4 on CD4⁺CD25⁺ Tregs may not take part in the immunopathogenesis of chronic HBV infection. In a word, this direct immunomodulation pathway of TLR2/4 on CD4⁺CD25⁺ Tregs may therefore provide potential applications in the treatment of CHB. Current data is only shown at a 1:1 ratio, which would not occur in nature, however, TLR2 and TLR4 has been proved to augment the functions of Tregs in this study. Also, the specificity of TLRs agonists has not been fully established and they may have far reaching effects on the cells outside of their TLR expression profiles. In addition, the *in vitro* study may not have an *in vivo* equivalence. Thus, the mechanism of this interaction requires further investigated *in vivo* and molecular mechanisms for the regulation of CD4⁺CD25⁺ Tregs via TLRs also needs to clarify.

In summary, our findings demonstrate that patients with HBV infection exhibited significant up-regulation of TLR2 and TLR4 on circulating monocytes. Exposure to HBeAg and HBcAg is able to change the expression profiles of TLR2 and TLR4. Furthermore, a marked increase in CD4⁺CD25⁺CD127^{low/-} Tregs was observed in the peripheral blood of CHB patients. Both Pam3Csk4 (TLR2 agonist)- and LPS (TLR4 agonist)-activated CD4⁺CD25⁺ Tregs

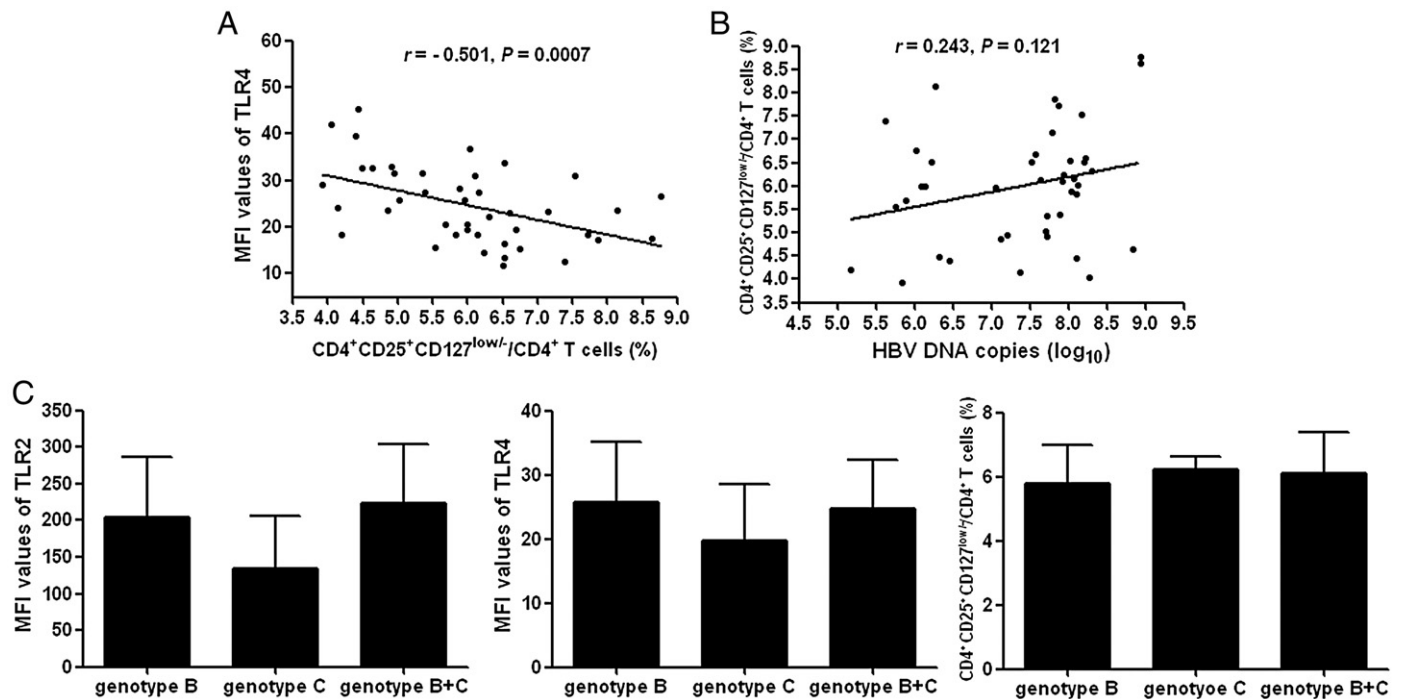


Fig. 4. Correlation analysis among Toll-like receptors (TLRs), regulatory T cells (Treg), and other factors in chronic hepatitis B (CHB) patients. Among the 42 CHB patients tested, correlation between TLR4 expression and circulating CD4⁺CD25⁺CD127^{low/-} Tregs (A), as well as between circulating CD4⁺CD25⁺CD127^{low/-} Tregs and serum HBV DNA load (B) were analyzed by Spearman correlation analysis. (C) TLR2, TLR4, and the frequencies of Tregs among different genotypes in CHB patients. The Kruskal–Wallis *H* test was performed for comparison between groups.

showed enhanced suppression function in CHBs. This study indicates a potentially important interaction between innate immune responses and immunoregulation during HBV infection. Further studies examining the mechanisms response for this interaction in the context of HBV infection will be critical and fruitful in guiding the development of new immunotherapeutics to treat CHB.

Materials and methods

Subjects

Blood samples were collected from 80 HBV-infected patients, including 16 patients with AHB, 42 patients with CHB, 22 of AsC. The standards for diagnoses were made according to the diagnostic standard of Chinese National Program for Prevention and Treatment of Viral Hepatitis. All patients were hospitalized or followed-up in Tangdu Hospital from May 2007 to June 2008. As a normal control, fresh blood samples were obtained from 20 healthy age and sex matched individuals. Patients who were co-infected with HIV, other hepatitis viruses, and any bacterial infection or concurrently of afflicted by immunocompromised diseases and autoimmune diseases were excluded. No patients received anti-HBV agents or immunomodulatory treatments for 6 months before sampling. The study protocol was approved by the ethics committee of Fourth Military Medical University, and written informed consent was obtained from each subject.

Virological assessment

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were determined by commercial enzyme immunoassay kits (Kehua Biotech, Shanghai, China). Serum HBV DNA was quantitated using a commercial real-time polymerase chain reaction (PCR) kit (PG Biotech, Shenzhen, China), with detection limit threshold of 500 copies/mL. HBV genotyping was performed as described previously (Naito, Hayashi, and Abe, 2001).

Isolation of peripheral blood mononuclear cells (PBMCs) and CD4⁺CD25⁺ Tregs

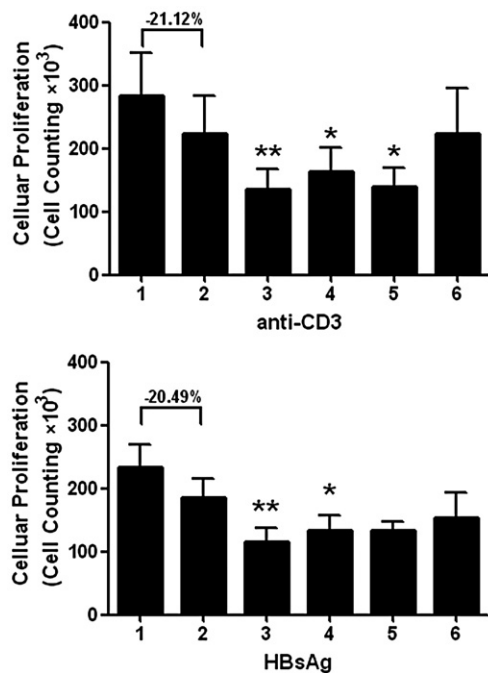
PBMCs were isolated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. CD4⁺CD25⁺ Tregs were purified using MagSelect Human CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (R&D systems, Huntingdon Valley, PA) according to the manufacturer's instruction. The purity of enriched cells for CD4⁺CD25⁺ T cells (>90%) was determined by flow cytometry. Cells were incubated in RPMI 1640 (Hyclone, Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen GIBCO, Grand Island, NY). PBMCs were cultured (10⁶/well) in 24-well plates with or without HBeAg (ViroStat, Portland, ME; 2 µg/mL) or HBcAg (AbD Serotec, Oxford, UK; 1 µg/mL) in different wells at 37 °C in a 5% CO₂ environment.

Flow cytometric analysis

PBMCs were harvested by centrifugation at 300 × g for 10 min at 4 °C. To stain TLR2 and TLR4, CD14-FITC (eBioscience, San Diego, CA), TLR2-PE (eBioscience) and TLR4-APC (eBioscience) monoclonal antibodies (mAbs) were utilized. To determine the frequencies of Tregs, mAbs CD4-PerCP (BD Bioscience, San Jose, CA), CD25-FITC (BD Bioscience) and CD127-PE (eBioscience) were used. Cells were incubated and stained at 4 °C in the dark for 30 min. Samples were analyzed with a four-color FACSCalibur analyzer (BD Biosciences Immunocytometry Systems). Acquisitions were performed with CellQuest Pro software (BD Biosciences Immunocytometry Systems) and analyses were performed with FlowJo version 5.7.2 for Windows (Tree Star Inc., Ashland, OR). Isotype control antibodies were used to separate positive and negative cells in the PerCP, FITC, PE, and APC fluorescence channels.

Suppression assay of CD4⁺CD25⁺ Tregs in response to TLR2 and TLR4 agonists

To evaluate the suppression activity, isolated CD4⁺CD25⁺ T cells were cultured together with autologous PBMCs-Tregs at a ratio of 1:1



1. PBMCs-Tregs
2. PBMCs-Tregs + Tregs
3. PBMCs-Tregs + Tregs (LPS activated)
4. PBMCs-Tregs + Tregs (Pam3Csk4 activated)
5. PBMCs-Tregs (LPS activated) + Tregs
6. PBMCs-Tregs (Pam3Csk4 activated) + Tregs

* $P < 0.05$ compared with lane 2

** $P < 0.01$ compared with lane 2

Fig. 5. Inhibitory capability of circulating CD4⁺CD25⁺ Tregs and TLR2/4 modulate on CD4⁺CD25⁺ Tregs. Blood was sampled from 10 patients with CHB. PBMC-Treg (depletion of Treg), PBMC-Treg + Treg (depletion of Treg and add back Treg at a ratio of 1:1) were used as effector cells, respectively. The effector cells were inoculated in 96-well plate at 100,000 cells per well, and stimulated by 10 µg/mL HBsAg or 1 µg/mL anti-CD3 Ab. The effects of Pam3Csk4 (TLR2 agonist) and LPS (TLR4 agonist) on both Tregs and PBMCs-Tregs were measured on cellular proliferation. Cells were cultured for 5 days. For the last 4 h, the cultures were pulsed with 10% of CCK-8. All data are shown as the mean ± standard deviation for 10 subjects in each group. The significance of differences was calculated using Dunn's multiple comparison test.

(5×10^4 cells, respectively), and stimulated with 1 µg/mL anti-CD3 (eBioscience) or 10 µg/mL HBsAg (AbD Serotec) in 96-well plates. Before mixture, Pam3Csk4 (InvivoGen, San Diego, CA) (400 ng/mL) or LPS (InvivoGen) (100 ng/mL) were added to the culture medium of CD4⁺CD25⁺ Tregs and PBMCs-Tregs, and the culture was maintained for 24 h and then washed twice with PBS. For proliferation test, the plate was cultured for 5 days, with replenishment of fresh medium containing 20 U/mL recombinant human (rh) IL-2 (Sigma) at day 3. Cellular proliferation was measured by Cell Counting Kit-8 (CCK-8, Alexis Biochemicals, San Diego, CA). Briefly, for the last 4 h culture, 20 µL (10% of the total volume) of CCK-8 solution were added to each well. Wells that contained known number of viable PBMCs were also prepared for creating a calibration curve. The absorbance of the samples was measured at 450 nm using Model 680 Microplate Reader (Bio-Rad, Hercules, CA).

Statistic analyses

Data were analyzed using SPSS version 13.0 for Windows. The Kruskal–Wallis H test and Dunn's multiple comparison test were used for comparison between groups. The Wilcoxon matched pairs test was

used to compare TLRs expression before and after antigen stimulation. Spearman correlation analysis was performed for correlation analysis. A value of $P < 0.05$ was considered to indicate a significant difference.

Acknowledgments

We thank the volunteers who generously participated in this study. We also thank Dr. Antonio Bertoletti (Infection and Immunity Program, Singapore Institute for Clinical Sciences, A*STAR, Singapore) for the enlightened discussion and critical review of the manuscript. This work was supported by the grant from National Key Technologies Research and Development Program of China during the 11th Five-year Plan Period (2008ZX10002-006).

References

- Akira, S., Takeda, K., 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4 (7), 499–511.
- Barboza, L., Salmen, S., Goncalves, L., Colmenares, M., Peterson, D., Montes, H., Cartagirone, R., Gutierrez Mdel, C., Berrueta, L., 2007. Antigen-induced regulatory T cells in HBV chronically infected patients. *Virology* 368 (1), 41–49.
- Boettler, T., Spangenberg, H.C., Neumann-Haefelin, C., Panther, E., Urbani, S., Ferrari, C., Blum, H.E., von Weizsacker, F., Thimme, R., 2005. T cells with a CD4⁺CD25⁺ regulatory phenotype suppress in vitro proliferation of virus-specific CD8⁺ T cells during chronic hepatitis C virus infection. *J. Virol.* 79 (12), 7860–7867.
- Cao, D., Malmstrom, V., Baecher-Allan, C., Hafler, D., Klareskog, L., Trollmo, C., 2003. Isolation and functional characterization of regulatory CD25^{bright}CD4⁺ T cells from the target organ of patients with rheumatoid arthritis. *Eur. J. Immunol.* 33 (1), 215–223.
- Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., Demengeot, J., 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197 (4), 403–411.
- Chen, Z., Cheng, Y., Xu, Y., Liao, J., Zhang, X., Hu, Y., Zhang, Q., Wang, J., Zhang, Z., Shen, F., Yuan, Z., 2008. Expression profiles and function of Toll-like receptors 2 and 4 in peripheral blood mononuclear cells of chronic hepatitis B patients. *Clin. Immunol.* 128 (3), 400–408.
- Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. *Annu. Rev. Immunol.* 13, 29–60.
- Dunham, R.M., Cervasi, B., Brenchley, J.M., Albrecht, H., Weintrob, A., Sumpter, B., Engram, J., Gordon, S., Klatt, N.R., Frank, I., Sodora, D.L., Douek, D.C., Paiardini, M., Silvestri, G., 2008. CD127 and CD25 expression defines CD4⁺ T cell subsets that are differentially depleted during HIV infection. *J. Immunol.* 180 (8), 5582–5592.
- Franzese, O., Kennedy, P.T., Gehring, A.J., Gotto, J., Williams, R., Maini, M.K., Bertoletti, A., 2005. Modulation of the CD8⁺ T-cell response by CD4⁺ CD25⁺ regulatory T cells in patients with hepatitis B virus infection. *J. Virol.* 79 (6), 3322–3328.
- Guo, Y.W., Wei, X.Q., Yang, S.J., 2007. [A close relationship between viral hepatitis B and Toll-like receptor 2]. *Zhonghua Gan Zang Bing Za Zhi* 15 (7), 485–488.
- Hartigan-O'Connor, D.J., Poon, C., Sinclair, E., McCune, J.M., 2007. Human CD4⁺ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. *J. Immunol. Methods* 319 (1–2), 41–52.
- Heeg, M.H., Ulsenheimer, A., Gruner, N.H., Zachoval, R., Jung, M.C., Gerlach, J.T., Raziorrouh, B., Schraut, W., Horster, S., Kauke, T., Spannagl, M., Diepolder, H.M., 2009. FOXP3 expression in hepatitis C virus-specific CD4⁺ T cells during acute hepatitis C. *Gastroenterology* 137 (4), 1280–1288 e1–6.
- Hoofnagle, J.H., Doo, E., Liang, T.J., Fleischer, R., Lok, A.S., 2007. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 45 (4), 1056–1075.
- Kinter, A.L., Hennessey, M., Bell, A., Kern, S., Lin, Y., Daucher, M., Planta, M., McGlaughlin, M., Jackson, R., Ziegler, S.F., Fauci, A.S., 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J. Exp. Med.* 200 (3), 331–343.
- Kondo, Y., Kobayashi, K., Ueno, Y., Shiina, M., Niitsuma, H., Kanno, N., Kobayashi, T., Shimosegawa, T., 2006. Mechanism of T cell hyporesponsiveness to HBeAg is associated with regulatory T cells in chronic hepatitis B. *World J. Gastroenterol.* 12 (27), 4310–4317.
- Liu, G., Zhao, Y., 2007. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4⁺ CD25⁺ T cells. *Immunology* 122 (2), 149–156.
- Liu, H., Komai-Koma, M., Xu, D., Liew, F.Y., 2006a. Toll-like receptor 2 signaling modulates the functions of CD4⁺ CD25⁺ regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* 103 (18), 7048–7053.
- Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St Groth, B., Clayberger, C., Soper, D.M., Ziegler, S.F., Bluestone, J.A., 2006b. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J. Exp. Med.* 203 (7), 1701–1711.
- Lok, A.S., McMahon, B.J., 2001. Chronic hepatitis B. *Hepatology* 34 (6), 1225–1241.
- Longhi, M.S., Mistry, R.R., Samyn, M., Scalori, A., Hussain, M.J., Quaglia, A., Mieli-Vergani, G., Ma, Y., Vergani, D., 2009. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. *Hepatology* 50 (1), 130–142.

- Medzhitov, R., Preston-Hurlburt, P., Janeway Jr, C.A., 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388 (6640), 394–397.
- Naito, H., Hayashi, S., Abe, K., 2001. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. *J. Clin. Microbiol.* 39 (1), 362–364.
- Pasare, C., Medzhitov, R., 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299 (5609), 1033–1036.
- Peng, G., Guo, Z., Kiniwa, Y., Voo, K.S., Peng, W., Fu, T., Wang, D.Y., Li, Y., Wang, H.Y., Wang, R.F., 2005. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* 309 (5739), 1380–1384.
- Peng, G., Li, S., Wu, W., Sun, Z., Chen, Y., Chen, Z., 2008. Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. *Immunology* 123 (1), 57–65.
- Riordan, S.M., Skinner, N., Kurtovic, J., Locarnini, S., Visvanathan, K., 2006. Reduced expression of toll-like receptor 2 on peripheral monocytes in patients with chronic hepatitis B. *Clin. Vaccine Immunol.* 13 (8), 972–974.
- Shen, L.S., Wang, J., Shen, D.F., Yuan, X.L., Dong, P., Li, M.X., Xue, J., Zhang, F.M., Ge, H.L., Xu, D., 2009. CD4(+)CD25(+)CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. *Clin. Immunol.* 131 (1), 109–118.
- Stoop, J.N., van der Molen, R.G., Baan, C.C., van der Laan, L.J., Kuipers, E.J., Kusters, J.G., Janssen, H.L., 2005. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 41 (4), 771–778.
- Sutmoller, R.P., den Brok, M.H., Kramer, M., Bennink, E.J., Toonen, L.W., Kullberg, B.J., Joosten, L.A., Akira, S., Netea, M.G., Adema, G.J., 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 116 (2), 485–494.
- Visvanathan, K., Skinner, N.A., Thompson, A.J., Riordan, S.M., Sozzi, V., Edwards, R., Rodgers, S., Kurtovic, J., Chang, J., Lewin, S., Desmond, P., Locarnini, S., 2007. Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein. *Hepatology* 45 (1), 102–110.
- Webster, G.J., Reignat, S., Brown, D., Ogg, G.S., Jones, L., Seneviratne, S.L., Williams, R., Dusheiko, G., Bertolotti, A., 2004. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. *J. Virol.* 78 (11), 5707–5719.
- Wei, X.Q., Guo, Y.W., Liu, J.J., Wen, Z.F., Yang, S.J., Yao, J.L., 2008. The significance of Toll-like receptor 4 (TLR4) expression in patients with chronic hepatitis B. *Clin. Invest. Med.* 31 (3), E123–E130.
- Wu, J., Lu, M., Meng, Z., Trippler, M., Broering, R., Szczeponek, A., Krux, F., Dittmer, U., Roggendorf, M., Gerken, G., Schlaak, J.F., 2007. Toll-like receptor-mediated control of HBV replication by nonparenchymal liver cells in mice. *Hepatology* 46 (6), 1769–1778.
- Xu, D., Fu, J., Jin, L., Zhang, H., Zhou, C., Zou, Z., Zhao, J.M., Zhang, B., Shi, M., Ding, X., Tang, Z., Fu, Y.X., Wang, F.S., 2006. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J. Immunol.* 177 (1), 739–747.
- Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K., Steinman, R.M., 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* 198 (2), 235–247.
- Yang, G., Liu, A., Xie, Q., Guo, T.B., Wan, B., Zhou, B., Zhang, J.Z., 2007. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. *Int. Immunol.* 19 (2), 133–140.
- Yu, X.L., Cheng, Y.M., Shi, B.S., Qian, F.X., Wang, F.B., Liu, X.N., Yang, H.Y., Xu, Q.N., Qi, T.K., Zha, L.J., Yuan, Z.H., Ghildyal, R., 2008. Measles virus infection in adults induces production of IL-10 and is associated with increased CD4+ CD25+ regulatory T cells. *J. Immunol.* 181 (10), 7356–7366.
- Yuh, C.H., Chang, Y.L., Ting, L.P., 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J. Virol.* 66 (7), 4073–4084.